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Regulation of Antibiotic Production by Bacterial Hormones

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REGULATION OF ANTIBIOTIC PRODUCTION BY BACTERIAL HORMONES

Nai-Hua Hsiao, Marco Gottelt, and Eriko Takano

Contents

1. Introduction	144
2. Rapid Small-Scale γ -Butyrolactone Purification	145
3. Antibiotic Bioassay	146
4. Kanamycin Bioassay	148
5. Identification of γ -Butyrolactone Receptors	150
6. Identification of the γ -Butyrolactone Receptor Targets	151
7. Gel Retardation Assay to Detect Target Sequences of the γ -Butyrolactone Receptors	152
7.1. Labeling of DNA fragments	153
8. Conclusions	156
Acknowledgments	156
References	156

Abstract

Antibiotic production is regulated by numerous signals, including the so-called bacterial hormones found in antibiotic producing organisms such as *Streptomyces*. These signals, the γ -butyrolactones, are produced in very small quantities, which has hindered their structural elucidation and made it difficult to assess whether they are being produced. In this chapter, we describe a rapid small-scale extraction method from either solid or liquid cultures in scales of one plate or 50 ml of medium. Also described is a bioassay to detect the γ -butyrolactones by determining either the production of pigmented antibiotic of *Streptomyces coelicolor* or kanamycin resistant growth on addition of the γ -butyrolactones. We also describe some insights into the identification of the γ -butyrolactone receptor and its targets and also the gel retardation conditions with three differently labeled probes.

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1. INTRODUCTION

Most secondary metabolites (including antibiotics) are the products of complex biosynthetic pathways activated in the stationary phase or during slow growth, which triggers the transition from primary to secondary metabolism (Bibb, 2005). This switch is complex and poorly understood, and involves many signals, including those by small signaling molecules called γ -butyrolactones. These signaling molecules, found mainly in *Streptomyces* species, are considered to be “bacterial hormones” because they are important in the regulation of antibiotic production and in some cases morphological differentiation (Takano, 2006). The first γ -butyrolactone, A-factor, was identified from *Streptomyces griseus* in 1967 and was isolated as a compound that could stimulate the production of streptomycin and sporulation in a mutant defective in these characteristics (Khokhlov *et al.*, 1967) (Fig. 6.1). Subsequently, the group of Sueharu Horinouchi identified the A-factor receptor (Onaka *et al.*, 1995) and the gene involved in the

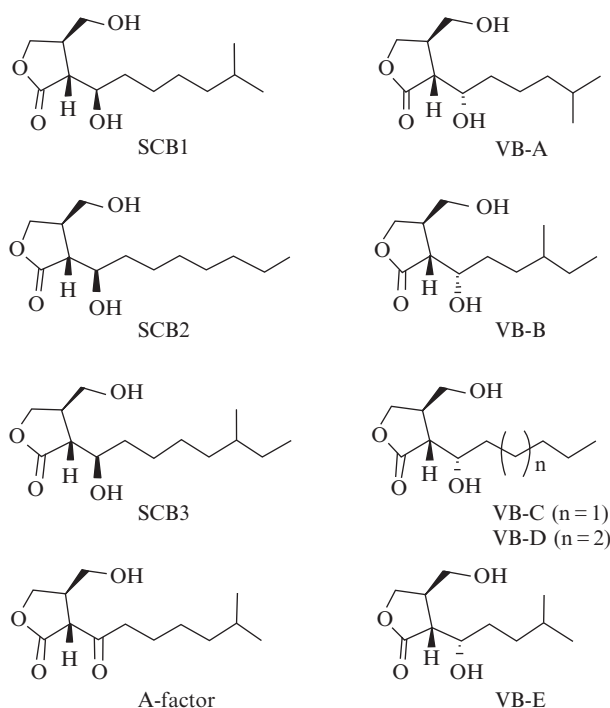


Figure 6.1 Chemical structures of the γ -butyrolactones. SCB1, 2, and 3 are produced by *S. coelicolor*; A-factor is produced by *S. griseus*; VB-A, B, C, D, and E are produced by *S. virginiae*. Figure contributed by Christian Hertweck with permission.

synthesis of A-factor (Horinouchi *et al.*, 1985) and also the pathway by which A-factor regulates streptomycin production (Ohnishi *et al.*, 1999). Other γ -butyrolactones and its receptor systems were identified in *Streptomyces virginiae* (Yamada, 1999 review), *Streptomyces lavendulae* FRI-5 (Kitani *et al.*, 2008), and *Streptomyces coelicolor* (Takano *et al.*, 2005), the model organism for *Streptomyces* genetics.

The number of identified receptor homologues has more than tripled in recent years with the analysis of antibiotic biosynthetic gene cluster sequences, suggesting that the γ -butyrolactone system may be a widespread regulatory component in controlling antibiotic production in the genus *Streptomyces* and in other actinomycetes (Takano, 2006).

These signaling molecules are produced only in minute concentrations, making them challenging to purify. However, their activity in nanomolar concentration is helpful in bioactivity assays, which can be performed with small quantities of material. In all the systems that have been reported, the γ -butyrolactones have been detected by such bioassays. For example: A-factor in *S. griseus* is extracted with chloroform and detected by measuring the induction of streptomycin production on a solid culture of a non-A-factor producing mutant, *S. griseus* HHI (Hara and Beppu, 1982); virginiae butanolides (VBs) in *Streptomyces virginiae* are extracted with ethyl acetate from acidified cell-free supernatants of cultures and detected by measuring the production of virginiamycin in a liquid culture of an *S. virginiae* mutant (Nihira *et al.*, 1988); IM-2 in *S. lavendulae* FRI-5 is extracted in the same way as VBs and detected by the induction of a blue pigment (Sato *et al.*, 1989).

To quickly assess whether γ -butyrolactones are a regulatory component in the antibiotics of choice, a large-scale purification or structural elucidation is not the first preference. This chapter describes the rapid small-scale extraction and detection of γ -butyrolactones in *S. coelicolor* which will aid in solving this problem. We also aim to aid identification of γ -butyrolactone receptors and their targets.

2. RAPID SMALL-SCALE γ -BUTYROLACTONE PURIFICATION

Intracellularly produced γ -butyrolactones are believed to diffuse passively out of the mycelium. In most protocols, liquid culture supernatants are used for production. However, the amounts of γ -butyrolactones produced in liquid cultures vary with the growth of the mycelium and extraction can be tedious. Therefore, we have developed a method using plates of solid agar medium. Here, we describe extraction protocols for both liquid and solid cultures.

1. Grow the strain either on plates up to aerial mycelium formation or early spore formation, or in liquid medium up to early stationary phase.

Note: Both minimal and rich media can be used in both cases; however, the stability of the γ -butyrolactones in rich media is much less than in a defined medium and they must be extracted at an earlier time.

2. For plates, cut the agar into large pieces and place in a beaker. Add 30–40 ml ethyl acetate to cover the agar pieces, and shake gently. Transfer the ethyl acetate into a rotary flask.

Note: Avoid breaking the agar into small pieces as this makes it difficult to separate the agar from the solvent. Also, it is important to test the ethyl acetate used for extraction beforehand as some ethyl acetate may already have bioactivity.

3. For liquid cultures, add a 2–3-fold volume of ethyl acetate in a separation flask. Shake 5–10 times, then separate the ethyl acetate phase from the water phase and place the ethyl acetate phase in a rotary flask.

Note: If a small amount of second phase separates from the ethyl acetate sample, remove it before evaporation. Normally, any second phase consists of fatty acids which cannot be evaporated will affect the diffusion ability of samples in the bioassay.

4. Evaporate all the ethyl acetate in a rotary evaporator and resuspend in 100% HPLC-grade methanol.

Note: From 3 plates or from 50 ml cultures, resuspend sample in $\leq 50 \mu\text{l}$ of methanol.

3. ANTIBIOTIC BIOASSAY

The classical approach for the bioassay of γ -butyrolactones is the observation or measurement of induced antibiotic production from an indicator strain in response to the compound of interest. Based on this concept, the antibiotic bioassay in *S. coelicolor* detects early production of the pigmented antibiotics, blue actinorhodin, and red prodiginines (Takano *et al.*, 2000).

1. Prepare spore stocks of the antibiotic bioassay indicator strain *S. coelicolor* M145 (Kieser *et al.*, 2000). Determine viable counts and store spores at -20°C in 20% glycerol.
2. Prepare supplemented minimal media solid (SMMS) plates (20 ml agar/plate; Takano *et al.*, 2001).

Note: The source of the agar strongly affects the timing and production of the pigmented antibiotics. Try to use only agar from the same company and the same batch.

3. For each plate, add 1×10^7 – 10^8 M145 spores (so the plate is just about confluent) in 60–100 μl of water. Using wet sterile cotton buds, spread confluent onto the plates and dry in the laminar flow cabinet.
4. Spot a sample ($<2 \mu\text{l}$) in the middle of the plate and dry. Incubate plates at 30 °C, check them every 8–12 h and record the antibiotic production by scanning the plates on a scanner (or take a photograph) (Fig. 6.2).

Note:

- Avoid dropping a sample before a plate is completely dry or dropping more than 2 μl of the sample. If the sample volume is more than 2 μl , spot in 2 μl aliquots several times after drying each time or concentrate the sample before spotting.
- The extracted samples should be spotted onto the indicator plate within 8 h of spreading, the sooner the better. No induction of the pigmented antibiotics will be observed if the indicator is grown for more than 8 h.
- The optimal amount of the positive control is 0.25 μg for the γ -butyrolactone SCB1. When using extracts made from *S. coelicolor* M145, 20–30 μl out of the 50 μl of sample either from 50 ml liquid cultures or from three plates should give bioactivity.
- To measure the approximate concentration of γ -butyrolactones isolated: for plates start the inoculum with the exact same number of viable spore counts for each sample; for liquid cultures measure the growth phase (by OD450 nm) convert into milligrams per dry weight and also measure the volume of the supernatant; this amount then can be used for comparison between samples.
- As seen in Fig. 6.2, the color may change depending on the amount of indicator spores used and also the length of time incubated.

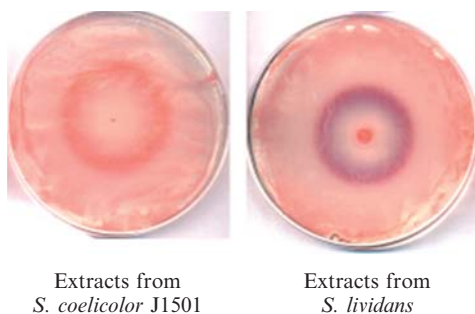


Figure 6.2 An example of a typical bioassay. Extracts from solid plates of *S. coelicolor* J1501 and *S. lividans* were spotted onto the indicator strain M145, then incubated for 36 h at 30 °C. The pigmented halos are the result of the butyrolactones stimulating pigmented antibiotic production.

4. KANAMYCIN BIOASSAY

The kanamycin bioassay was developed as an easy and direct method to detect γ -butyrolactone. It is based on the γ -butyrolactone receptor, ScbR, and its binding to the target DNA and the γ -butyrolactones. ScbR represses transcription of its own gene and that of *cpkO*, a pathway-specific regulatory gene for the Cpk cluster, by binding to the promoter regions (Pawlik *et al.*, 2007; Takano *et al.*, 2005). This repression is abolished by SCB1, resulting in transcription of the target genes. Using these principles, a γ -butyrolactone detection plasmid, pTE134, was constructed which harbors *scbR* and its own promoter region with the ScbR binding sites, together with the *cpkO* promoter with the ScbR binding sites, fused to a promoterless-kanamycin resistance gene (Fig. 6.3). pTE134, was transferred into *S. coelicolor* LW16 (*scbA* and *scbR* double deletion mutant) to obtain the kanamycin indicator strain LW16/pTE134. LW16 was chosen as a host strain for the kanamycin bioassay because it does not produce γ -butyrolactones and it lacks *scbR*, thus avoiding competition from endogenous ScbR. The kanamycin bioassay indicator strain LW16/pTE134 is sensitive to kanamycin but when γ -butyrolactones are added the repression caused by ScbR is abolished

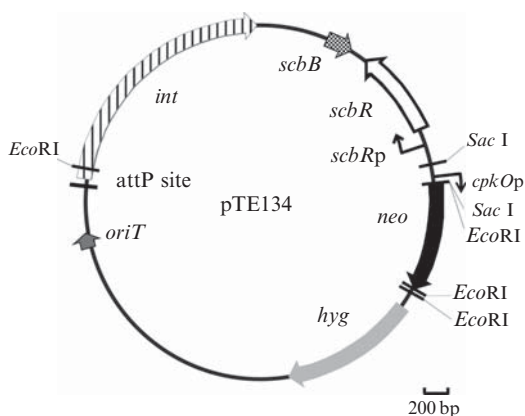


Figure 6.3 Schematic map of pTE134 used for the kanamycin bioassay. pTE134 has *scbR* (open arrow) with its own promoter region, *scbRp* (black arrow) and a *cpkO* promoter, *cpkOp* (black arrow) coupled with a promoterless kanamycin resistance gene (*neo*, solid arrow). The light gray arrow indicates *hyg* (omega hygromycin resistance gene), the deep gray arrow represents *oriT* (RP4 origin of single-stranded DNA transfer), the hatched arrow represents *int* (phiC31 integrase), the shaded arrow indicates a partial coding region (204 bp) of *scbB* and the attP site (phage phiC31 attP site) is indicated by a vertical black line. Restriction sites used for cloning are indicated by black lines (not all *SacI* and *EcoRI* sites are shown on the map).

and the kanamycin resistance gene is transcribed, rendering the indicator strain kanamycin resistant (Hsiao *et al.*, submitted for publication).

This assay was also tested for its ability to detect other butyrolactones than the ones produced by *S. coelicolor*. Though the sensitivity is weaker than for the native γ -butyrolactones, with enough material spotted to the plates, kanamycin resistance can be observed. Furthermore, γ -butyrolactones have been identified from those antibiotic producing *Streptomyces* strains which were not known to produce these molecules using this assay (Hsiao *et al.*, submitted for publication).

1. Prepare spore stocks of the kanamycin bioassay indicator strain LW16/pTE134 and stock spores in 50–100 μ l aliquots at -20°C in 20% glycerol.

Note: Because this indicator strain is unstable, spores need to be collected from MS plates (Kieser *et al.*, 2000) containing 50 $\mu\text{g}/\text{ml}$ hygromycin, then aliquoted. Avoid thawing and refreezing the spore stocks.

2. Prepare fresh DNAgar (Difco Nutrient Agar) plates containing kanamycin at a final concentration 3–5 $\mu\text{g}/\text{ml}$ (20 ml per plate).

Note: Because the *cpkO* promoter has very low activity (conferring resistance to <10 $\mu\text{g}/\text{ml}$ kanamycin), the concentration of kanamycin should not be higher than 5 $\mu\text{g}/\text{ml}$ (Fig. 6.4).

3. To each plate, add 2.6×10^6 spores of the indicator strain in about 60–100 μ l of water. Using wet sterile cotton buds, spread confluentlly onto the plates and dry.
4. Spot the extract or sample (<2 μ l) in the middle of the plate and dry. Incubate plates at 30°C , check them after 2–3 days and record the growth by scanning the plates on a scanner. The kanamycin resistant colonies will grow around the spot where the γ -butyrolactones have diffused.

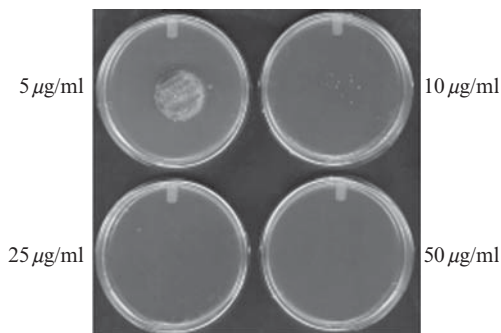


Figure 6.4 The kanamycin bioassay using different kanamycin concentrations in the indicator media. 0.1 μg of chemically synthesized SCB1 was spotted onto lawns of LW16/pTE134 (2.6×10^6 spores) on DNAgar plates containing 5, 10, 25, or 50 $\mu\text{g}/\text{ml}$ kanamycin, respectively. The plates were incubated at 30°C for 3 days.

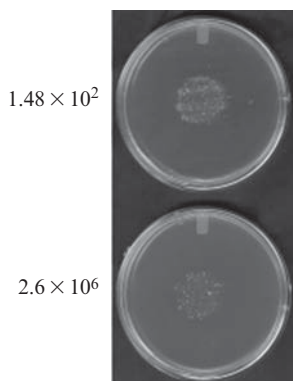


Figure 6.5 The kanamycin bioassay using different amount of indicator strains. $0.1\ \mu\text{g}$ of chemically synthesized SCB1 was spotted onto lawns of LW16/pTE134 (2.6×10^6 or 1.48×10^7 spores) on DNAgar plates containing $5\ \mu\text{g}/\text{ml}$ kanamycin, respectively. The plates were incubated at $30\ ^\circ\text{C}$ for 3 days.

Note:

- Again avoid spotting a sample before a plate is completely dry or spotting more than $2\ \mu\text{l}$ of the sample. If the sample volume is more than $2\ \mu\text{l}$, spot in $2\ \mu\text{l}$ aliquots several times after drying each time or concentrate the sample before spotting.
- The optimal amount of the positive control is $0.1\ \mu\text{g}$ for SCB1 and $0.25\ \mu\text{g}$ for A-factor. To enhance the density of the growth halo, a larger amount of indicator spores and a smaller amount of kanamycin in the indicator plate can be used (Figs. 6.4 and 6.5).

5. IDENTIFICATION OF γ -BUTYROLACTONE RECEPTORS

Several groups have used different methods to identify the γ -butyrolactone receptors. The first receptor to be identified was by Horinouchi and co-workers using an A-factor affinity column and recovering the receptor from the crude extract of *S. griseus* (Onaka *et al.*, 1995). The same technique was used to identify the γ -butyrolactone receptors from *S. virginiae* (Okamoto *et al.*, 1995) and *S. lavendulae* FRI-5 (Ruengjitchatchawalya *et al.*, 1995). Another approach was via PCR using degenerate oligonucleotides to isolate the two receptor homologues in *S. coelicolor* (Onaka *et al.*, 1998). Interestingly, Onaka and co-workers failed to identify ScbR with this approach. This may be due to the limited homologous sequence information at the time.

We have identified ScbR only by cloning the γ -butyrolactone synthase gene, ScbA, which lies divergent from the ScbR gene, using PCR (Takano *et al.*, 2001). In most of the γ -butyrolactone systems identified (apart from that in *S. griseus*) synthase and receptor genes are next to or very close to each other. It is best to use this property and to identify the γ -butyrolactone synthase gene first using degenerate oligonucleotides or by an *in silico* approach. The degenerate oligonucleotides should be designed near the active sites we have identified by mutagenesis analysis, which also correspond to the AfsA repeats (Hsiao *et al.*, 2007).

Another approach is to use any sequence information available. These γ -butyrolactone receptors will resemble TetR repressors and may be found especially concentrated close to antibiotic biosynthetic gene clusters. There are usually several γ -butyrolactone receptor homologues in one strain. To determine the “real” receptor, the amino acid identity should be more than 40% compared to the functionally proven γ -butyrolactone receptors, that is, ArpA, BarA, FarA, and ScbR. These proteins also will have a pI of around 5–6 compared to the other ScbR homologues whose pIs are much higher at around 9–11.

6. IDENTIFICATION OF THE γ -BUTYROLACTONE RECEPTOR TARGETS

The first target to be identified was from the A-factor system by use of the genomic SELEX (systematic evolution of ligands by exponential enrichment) system (Ohnishi *et al.*, 1999). This method uses purified γ -butyrolactone receptor in gel retardation assays on partially digested chromosomal DNA to find the binding sites. Several groups have now proposed the target sequences for the γ -butyrolactone receptors (Folcher *et al.*, 2001; Kinoshita *et al.*, 1999; Onaka and Horinouchi, 1997) (Fig. 6.6). However, these sequences are not sufficiently conserved compared with the identified targets for ScbR (Fig. 6.6).

We have used the properties of the TetR receptor family, which the γ -butyrolactone receptors resemble. TetR proteins regulate their own genes and/or the adjacent gene. By using this property, gel retardation was performed to identify one of the ScbR targets. To identify another target of ScbR, microarray analysis was performed, which did not result in the identification of a direct target but rather the downstream genes which represent the Cpk antibiotic biosynthetic gene cluster. But doing so, the direct target, a pathway-specific activator, for the Cpk cluster was identified. Surprisingly, of the four targets of ScbR that we have identified, two, site R and site OA, have 100% consensus sequences at 6 bp at each ends. While the

SiteA (<i>scbA/R</i> promoter region)	GAAAAA AAACCG CTCTAGTCTGT ATCTTAA
SiteOB (<i>kasO</i> promoter region)	CAA AAACAG ACTTGT TAGCTGTTT
SiteR (<i>scbA/R</i> promoter region)	GG AAACCGG CAATGCG GGTTTGT TCGATC
SiteOA (<i>kasO</i> promoter region)	ACA AAACCGG TGTGCT GGTTTGT AAAGTCGTGG
<i>ScbR</i> binding consensus	AAACCGGNNNNNNGGTTTGT
ArpA binding consensus	A ^A _{GT} AAACCG ^A _G CCGG ^T _C GGT ^{AT} _{TC} G ^T _G
BarA, FarA binding consensus	aCa ^{AA} _{TG} GAAC ^c _{CGGA} CGgtc ggTTTG
SpbR binding consensus	TNANA ^A _T CNNAC ^C _T NNNG GGTTT ^G _T TTT

Figure 6.6 Consensus sequences of different γ -butyrolactone receptors. The target sequences found so far for ScbR and its consensus sequences in *S. coelicolor* are presented on the top five lanes. The bottom three lanes are consensus sequences reported for the different receptor proteins. ArpA: Onaka and Horinouchi (1997); BarA, FarA: Kinoshita *et al.* (1999); SpbR: Folcher *et al.* (2001). Bold letters represent the conserved base pairs in ScbR binding sites. The base pairs in italic in siteA and siteOB represent the conserved base pairs between these two sites.

others, site A and site OB, only have 4 bp that are conserved with site R and site OA. In contrast, within the pair, 11 bp are conserved out of 23 bp (Fig. 6.6) (Takano *et al.*, 2005).

To identify the γ -butyrolactone receptor targets, first use the promoter region itself, and also include the promoter region of the gene adjacent to it, to perform a gel retardation assay with either a purified protein or a crude extract. The activity of the protein is stabilized when the protein is still in the crude extract and at a low concentration. As soon as the protein is purified and concentrated, it tends to aggregate and will no longer be suitable for any further experiments to analyze activity. Use the identified target sequence to identify further targets *in silico*.

7. GEL RETARDATION ASSAY TO DETECT TARGET SEQUENCES OF THE γ -BUTYROLACTONE RECEPTORS

Gel retardation is not a new technique and can be found described in many publications (e.g., Folcher *et al.*, 2001; Kinoshita *et al.*, 1999; Onaka and Horinouchi, 1997). However, we have experience in using three differently labeled γ -butyrolactone receptor target DNA fragments, and have optimized the technique for each probe using pure protein and crude extracts from either *Escherichia coli* or *Streptomyces*.

7.1. Labeling of DNA fragments

7.1.1. Radio-labeled probes

1. The oligo was first labeled at the 5' ends by incubation of 50 pmol of oligo, 5 μ l of [γ -³²P]ATP (10 mCi/ml), 1 μ l of $\times 10$ kinase buffer, and 1 μ l T4 nuclease kinase in a total volume of 50 μ l incubated at 37 °C for 30 min⁻¹ h. The reaction was stopped and cleaned by addition of $\times 1$ volume of phenol/chloroform with vortexing, then the phenol was extracted by addition of either $\times 1$ vol of chloroform or ether, twice. The oligo were precipitated using 100% EtOH with 3 M NaoAc and 1 μ g of glycogen at -20 °C preferably O/N. The mixture was spun at 4 °C for 30 min, then the supernatant discarded, and the pellet air dried.
2. PCR was conducted using labeled- and unlabeled-oligos. 60 ng of template, 50 pmol of both unlabeled- and labeled-oligos, 200 μ M dNTP, 5% DMSO, and PCR buffer (commercial) together with Taq polymerase were incubated to amplify in a total volume of 100 μ l. The PCR conditions are: 95 °C for 5 min, then 30 cycles of 95 °C for 50 s, 55 °C for 40 s, 72 °C for 40 s, then extend at 72 °C for 5 min. Five microliters of the reaction was loaded on a gel to assure amplification. If extra bands are amplified, the fragment of interest can be purified using a commercial gel-extraction kit. The final volume should be eluted in 50 μ l.
3. Gel retardation was conducted with 2.5 ng of labeled DNA (about 10 cpm), pure protein or 0–15 μ l crude extract from *E. coli* or *Streptomyces*, 125 mM HEPES pH 7.5, 20 mM DTT, 20% glycerol, 200 mM KCl, 0.16 μ g/ μ l calf thymus DNA in a final volume of 12.5–25 μ l. The mixture was incubated at room temperature for 10 min, then 2–4 μ l of loading dye (50%(v/v) glycerol, 0.25% (w/v) bromophenol blue, 10 mM Tris-HCl, pH 8, 1 mM EDTA) was added. To detect the binding of the receptor to the butyrolactones, the butyrolactones can be added either before or 10 min after the incubation. In the case of SCB1, 1 μ g was added to see a release of the full shift.

Note:

- For the *E. coli* crude extract, a 10 ml LB overnight preculture of *E. coli* harboring a plasmid with the γ -butyrolactone receptor gene under the control of the *lacZ* promoter was inoculated at a 1:100 dilution in 50 ml LB and grown at 37 °C until OD₆₀₀ 0.7–0.8 when induced with 1 mM (final conc.) IPTG (Isopropyl β -D-1-thiogalactopyranoside). After another 3 h of incubation, cells were harvested by centrifugation and the cell pellet was washed twice with buffer (50 mM of Tris pH 7.0, 1 mM of EDTA, 1 mM of DTT, 100 mM of phenylmethylsulfonylfluoride (PMSF)) and resuspended in 500 μ l of the same buffer. 100 μ l aliquots were stored at -80 °C for later use. For crude extract

preparation, the cells were immediately disrupted by sonication. The cell lysate was clarified by centrifugation and the supernatant used as a crude extract after determination of the total protein content.

- For the *S. coelicolor* crude extract, *S. coelicolor* was grown in 50 ml of SMM then the same procedure as above was followed except the cells were resuspended in 100 μ l prior to sonication and 10 μ l was used for the gel retardation analysis. Due to the growth phase-dependent expression of ScbR, the time point of growth when the cells were harvested is crucial to detect any protein binding to the DNA.
 - KCl was the best salt for this assay and MgCl was not suitable.
4. For detection of 100–300 bp DNA fragments, a 5% acrylamide gel (5% acrylamide: bisacrylamide = 37.5:1, 1.25 ml of $\times 10$ TBE, 15 μ l of TEMED, 87.5 μ l of 10% AMPS in a total of 12.5 ml) was used in $\times 1$ TBE (90 mM Tris–HCl, 90 mM boric acid, 2 mM EDTA, pH 8.0) running buffer. It is also possible to use a 3.5% acrylamide gel to detect a longer DNA fragment. A Bio-Rad Mini Protean kit was used for running the gel at 100 V constant for 1 h till the BPB just ran off the gel.
 5. The gel was then taken off the glass and wrapped in cling film (Saran wrap) and directly placed onto an X-ray film (Super RX (Fujifilm)), and exposed for 30 min at room temperature in a cassette. The film was developed using an automated film developer (Fig. 6.7A).

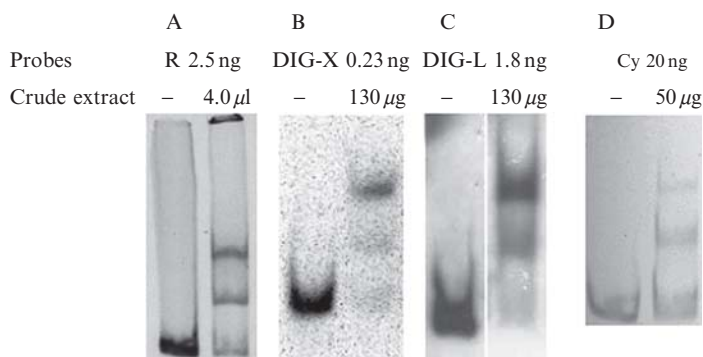


Figure 6.7 Gel retardation assay using differently labeled probes. Gel retardation using: (A) Radio-labeled probes (R); (B) DIG-labeled probes with detection using X-ray film (DIG-X); (C) DIG-labeled probes with detection using Lumi-imager (DIG-L); (D) Cy3-labeled probes (Cy). All probes were made using a 146 bp DNA fragment which includes the ScbR binding site R which is situated at –68 to –42 nt from the transcriptional start site (Takano *et al.*, 2001). The amount of each probe used is indicated next to each probe name. Crude extract (given in total protein as microl, microg) from *E. coli* harboring pIJ6120 was used and the amount is indicated on the top of the lanes.

7.1.2. DIG-labeled probes

1. DNA fragments were first amplified by PCR using both unlabeled-oligos using the PCR conditions mentioned in Point 2 of [Section 7.1.1](#). Purified PCR product (either by a cleanup kit or by extracting from a gel), 100 ng, was used in the DIG gel shift kit 2nd generation (Roche) labeling procedure, steps 5–7 (p11 of Roche protocol). (Note: this kit labels 3' ends.)
2. After labeling, the probes were incubated with the protein for gel retardation assays as in Point 3 of [Section 7.1.1](#).

Note:

- The labeled probes can be stored at -20°C for a very long time.
 - Depending on the sensitivity of the detection method used (see below), the amount of DIG-labeled probe may have to be varied. In our case, eightfold less probe was used for detection with X-ray films (we used 1.8 ng of labeled probe) compared to when a Lumi-imager F1 (we used 0.23 ng labeled probe) was used for detection ([Fig. 6.7B and C](#)).
 - After optimization of the amount of probe used, the protein concentration in the assay will have to be adjusted to keep the protein/DNA ratio constant.
3. After running, the gel was taken off the glass plates for direct contact-blotting (Roche protocol 3.7.2), further crosslinking (3.7.3), and chemiluminescent detection (3.8). The membrane was detected using an X-ray film exposed for 20 min, then developed in an automated X-ray film developer ([Fig. 6.7B](#)) or in a Lumi-imager F1 (Roche) for 40 min at room temperature ([Fig. 6.7C](#)).

7.1.3. Cy3-labeled probes

The DIG-labeling protocol will take up to 2 days. To improve the efficiency in time, Cy3-labeled oligos are now our preferred choice. A preliminary protocol is described below.

1. 5'-Cy3-labeled oligo was purchased from Sigma-Aldrich and used for PCR along with the unlabeled-oligo as in Point 3 of [Section 7.1.1](#). The amplified product was purified and the concentration measured by Nanodrop (Thermo scientific).
2. The gel retardation was conducted with 20 ng of Cy3-labeled DNA fragment with 50–300 μg of crude extracts from *Streptomyces* as in Points 3 and 4 of [Section 7.1.1](#). After running, the gel with the glass plate is exposed for 30 s and detected in a LAS 4000 (Fujifilm) ([Fig. 6.7D](#)).

Note: A fluorescence compound was detected in the *Streptomyces* crude extract which may interfere with detection.

8. CONCLUSIONS

The rapid small-scale purification of the γ -butyrolactones and the kanamycin assay have simplified the detection of the γ -butyrolactones, which are found to be one of the important factors in the regulation of antibiotic production. From genome sequences and also from the sequence of many antibiotic biosynthetic gene clusters many homologues to the γ -butyrolactone synthases and receptors have been identified, but the number of γ -butyrolactones elucidated has not increased since year 2000. This method may provide an easy solution to detect the γ -butyrolactones from those strains which have the synthase and receptor homologues and also to identify new organisms that may produce γ -butyrolactones. The binding sensitivity of the different γ -butyrolactones to ScbR has been tested and found to be lower; however, with enough material kanamycin resistance was observed (Hsiao *et al.*, submitted). Identification of the receptor target is a major job. However, with the several hints obtained from previous work and also from our experience with the gel retardation assays, it may be possible to quicken the procedure.

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